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THE EFFECT OF SALT REGIMENS ON THE DEVELOPMENT OF (Na^+-K^+)-DEPENDENT ATPase ACTIVITY DURING THE GROWTH OF SALT GLANDS OF DUCKLINGS

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SUMMARY

1. A Mg^{2+}-activated, ouabain-sensitive (Na^+-K^+)-ATPase and a ouabain-insensitive Mg^{2+}-ATPase were characterized in salt gland homogenates of the domestic duck.

2. 3 days after hatching, experimental birds were given 1% aqueous NaCl to drink for 12 h and fresh water for the remainder of each day. Control ducklings were maintained exclusively on fresh tap water. In homogenates of salt glands from experimental birds the specific activity of ATPase reached maximal levels in 9 days. (Na^+-K^+)-ATPase increased by a factor 4.1 and Mg^{2+}-ATPase increased by a factor of 1.8. There was no increase in ATPase activity in homogenates from control birds.

3. Homogenates of glands from birds drinking only fresh water, after being maintained on the salt-water regimen for 22 days, exhibited a logarithmic decrease in (Na^+-K^+)-ATPase activity and in 9 days reached the level of activity recorded for control birds maintained only on fresh water. The Mg^{2+}-ATPase activity decreased rather slowly for the first 5 days on fresh water, and then dropped rapidly during the last 4 days of the experiment.

4. The increase in ATPase levels in the salt glands of birds on the salt-water regimen is not cation-specific: both KCl and $MgCl_2$ regimens are capable of increasing this level, although neither is as effective as NaCl.

5. Differential rates of increase and decrease of (Na^+-K^+)-ATPase and Mg^{2+}-ATPase activity resulting from various salt diets suggest that these activities may not be a consequence of bimodal functioning of the same enzyme.

INTRODUCTION

10 years ago SKOU[1] demonstrated an ATPase (ATP phosphohydrolase, EC 3.6.1.3) in crab nerves which is activated by Mg^{2+}, Na^+ and K^+. Subsequent studies

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revealed the (Na^+-K^+)-ATPase in a wide variety of tissues[2-9]. The suggestion that this enzyme plays an integral role in ion transport[2] is supported by the observation that both active transport of Na^+ and K^+ and (Na^+-K^+)-ATPase activity have an obligatory requirement for Mg^{2+}, Na^+ and K^+ and are both inhibited by ouabain[2,9]. Details of this subject are reviewed by SKOU[10].

The salt glands of marine birds provide an excellent tissue for the study of the enzyme. These nasal glands function in extrarenal excretion in several species of marine birds[11-14]. This nasal secretion, which is hypertonic to plasma and sea water and which is under the control of adrenocorticosteroids[15] and cholinergic nerve fibers[16], is a response to a general osmotic load[11]. Since secretion is inhibited by a retrograde injection of ouabain[17], a (Na^+-K^+)-ATPase appears to be necessary for secretory function of the gland and HOKIN[8] and BONTING *et al.*[7] recently demonstrated the enzyme in homogenates of avian salt glands.

Although the enzyme is associated with ion transport, little is known about the onset of activity in developing systems. Since the salt gland undergoes hypertrophy[18] and histochemical differentiation[18,19] in response to osmotic stress, it is an excellent organ for such a study. Observations reported here suggest that the hypertrophy and differentiation of the salt gland parallels a four-fold increase in the specific activity of (Na^+-K^+)-ATPase. Moreover, the kinetics of this increase in activity are consistent with current views concerning enzyme induction.

MATERIALS

Animals

150 domestic ducklings, received 2 days after hatching, were placed on a diet consisting of duck starting mash and fresh water and raised under an artificial 12-h photoperiod. 2 days later (day zero) and for the next 31 days, half of the ducks (experimental group) were given a 1% solution of NaCl for 12 h and fresh tap water for the remainder of the day, while the remaining ducks (control group) received fresh tap water exclusively. In addition, 12 control birds were placed on the salt-water regimen on day 15 of the experiment and 16 experimental birds were placed on fresh water on day 22 of the experiment. Finally, two groups of birds were given either a 1.25% KCl or a 1.17% $MgCl_2$ solution to drink (these concentrations are approximately iso-osmotic with 1% NaCl).

Preparation of salt gland homogenates

On various days, four experimental and four control birds were weighed and then sacrificed by decapitation. The paired salt glands from each bird were extirpated, trimmed of extraneous connective tissue, weighed and then homogenized in 0.25 M sucrose (final concentration of 2.5 mg wet weight tissue per ml).

Assay of ATPase activity

Immediately after preparation, salt gland homogenates were assayed for ATPase activity. The assay system was a modification of that used by BONTING, SIMON AND HAWKINS[4]. Homogenates were assayed for both total ATPase activity and for ouabain-insensitive ATPase (Mg^{2+}-ATPase) activity as described in Fig. 1. Ouabain-sensitive ATPase ((Na^+-K^+)-ATPase) activity was determined by the

difference between these two activities. P_i was determined by the technique suggested by BONTING, SIMON AND HAWKINS[4] and trichloroacetic acid-precipitable protein was determined by the method of LOWRY *et al.*[20]. Enzyme activity is expressed as either mM P_i liberated per ml of homogenate per h or as mM P_i per mg protein per h. Disodium and Tris salts of ATP and ouabain were obtained from Sigma Chemical Co.

RESULTS

Kinetics of Mg^{2+}-ATPase and (Na^+-K^+)-ATPase

The per cent of the total ATPase activity represented by (Na^+-K^+)-ATPase varied in these experiments between 40 and 60%. Fig. 1 shows the effect of substrate concentration on the amount of phosphate liberated by Mg^{2+}-ATPase and by (Na^+-K^+)-ATPase. The K_m for both enzymes, determined by a double-reciprocal plot

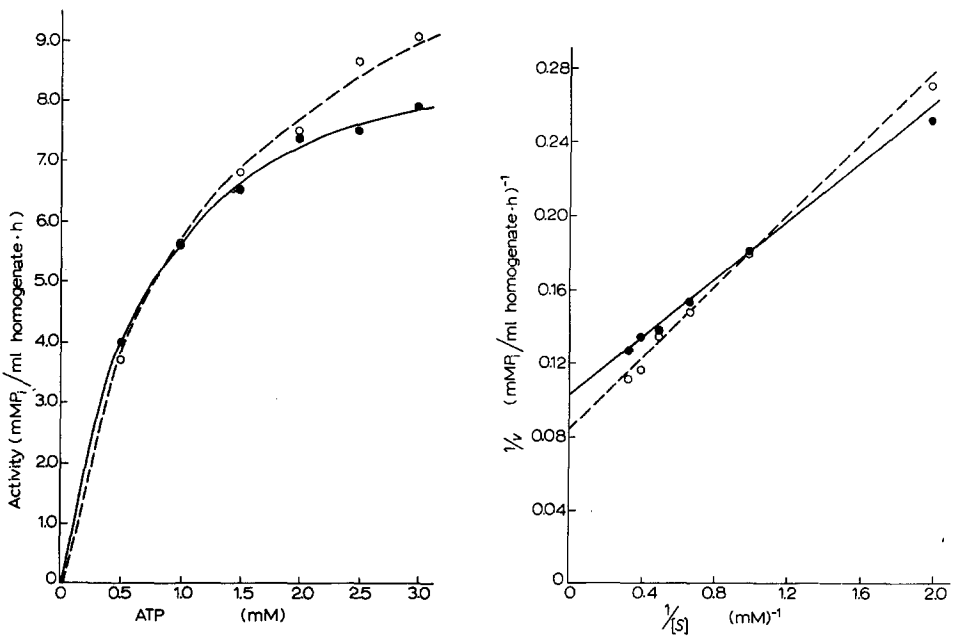


Fig. 1. Liberation of P_i as a function of ATP concentration. Total ATPase activity was determined in a 1.5-ml reaction volume containing 0.1 ml of a salt gland homogenate (2.5 mg wet weight tissue per ml 0.25 M sucrose) and the following components (final concentrations): 6 mM $MgCl_2$, 5 mM KCl, 60 mM NaCl, 92 mM Tris–HCl buffer (pH 7.2) and the disodium salt of ATP as shown. Mg^{2+}-ATPase activity (●—●) was determined in a reaction mixture which contained, in addition, 0.1 mM ouabain. (Na^+-K^+)-ATPase activity (○- - -○) is represented by the difference between these two activities. Incubation time was 15 min at 37°. The reaction was stopped by the addition of 2.5 ml of 20% trichloroacetic acid. All kinetic experiments were carried out with salt gland homogenates from ducks which had been maintained on salt water for several months.

Fig. 2. K_m determination of Mg^{2+}-ATPase (●—●) and (Na^+-K^+)-ATPase (○- - -○) from a double-reciprocal plot of the data shown in Fig. 1. The K_m was 0.74 mM for Mg^{2+}-ATPase and 1.13 mM for (Na^+-K^+)-ATPase. Maximum velocity for the two enzymes was 0.97 and 1.19 mM P_i per ml homogenate per h, respectively.

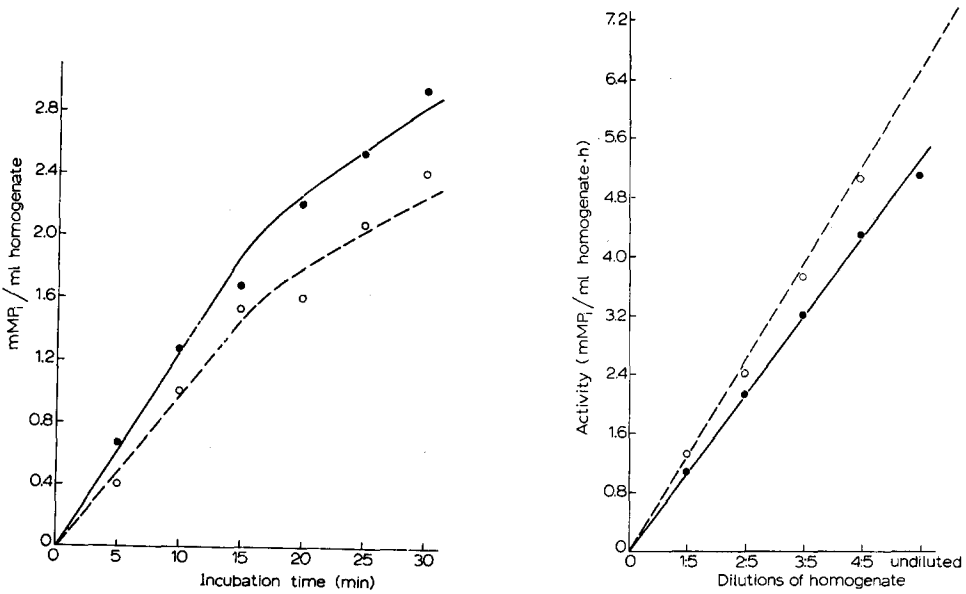


Fig. 3. Liberation of P_i as a function of incubation time. ATP concentration was 3 mM. Other conditions were the same as in Fig. 1. ●—●, Mg^{2+}-ATPase; ○- - - -○, (Na^+-K^+)-ATPase.

Fig. 4. Liberation of P_i as a function of enzyme concentration. 0.1 ml of the undiluted homogenate (2.5 mg wet weight tissue per ml of 0.25 M sucrose) and 0.1 ml of various dilutions of the homogenate were added to the reaction mixture described in Fig. 1. ATP concentration was 3 mM. ●—●, Mg^{2+}-ATPase; ○- - - -○, (Na^+-K^+)-ATPase.

(Fig. 2) of the data shown in Fig. 1, was essentially the same: 0.74 mM for Mg^{2+}-ATPase and 1.13 mM for (Na^+-K^+)-ATPase. The range of K_m values for Mg^{2+}-ATPase and (Na^+-K^+)-ATPase for three different homogenates was 0.74–0.94 mM and 0.75–1.22 mM, respectively. Fig. 3 shows that the rate of phosphate liberation was directly proportional to time over the first 15 min of the incubation period. Furthermore, the rate of the reaction for both enzymes was directly proportional to enzyme concentration (Fig. 4). The concentration of homogenate in all experiments was adjusted so that the total ATPase activity hydrolyzed less than 10% of the substrate during the 15-min incubation period.

Fig. 5 indicates that both enzymes have an absolute requirement for Mg^{2+} and that optimal activity occurs at an ATP/Mg^{2+} ratio of 1. When this ratio is less than 1, some inhibition is observed although this depressed level is constant at 4 mM Mg^{2+} or greater. A ratio of 3 ATP/6 Mg^{2+} was used in all experiments in order to reduce any variability in enzyme activity that might result from small deviations of the optimal ATP/Mg^{2+} ratio of 1.

In the presence of 5 mM K^+, addition of Na^+ caused an activation of ATPase activity which reached a maximum at 40 mM and remained unchanged up to 80 mM (Fig. 6). This activation by Na^+ was entirely ouabain-sensitive, whereas the ouabain-insensitive ATPase was unaffected by added Na^+. In the presence of 60 mM Na^+, addition of K^+ caused a similar activation which was ouabain-sensitive and which reached a plateau at 4 mM (Fig. 7). Half-maximal activation occurred at 0.8 mM K^+.

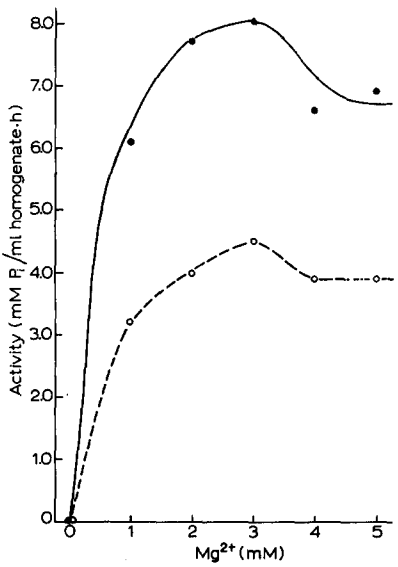
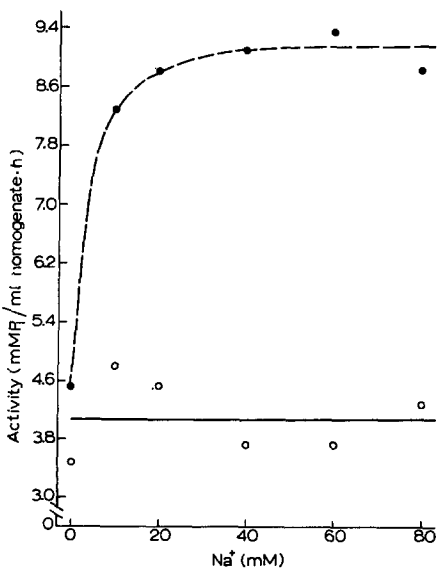


Fig. 5. Liberation of P_i as a function of Mg^{2+} concentration. ATP concentration was 3 mM. Other conditions were the same as in Fig. 1. ●—●, Mg^{2+}-ATPase; ○- - - -○, (Na^+-K^+)-ATPase.



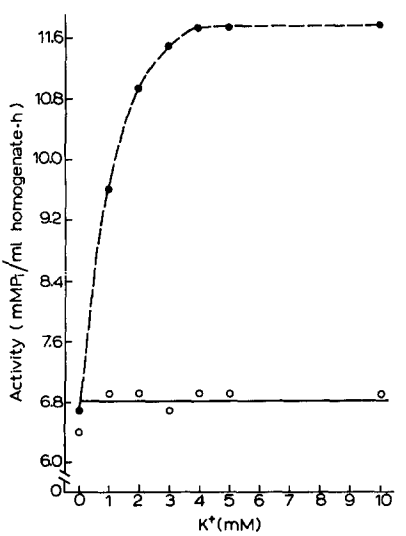


Fig. 6. Liberation of P_i as a function of Na^+ concentration in the presence of 5 mM K^+. The Tris salt of ATP was substituted for the disodium salt. ATP concentration was 3 mM. Other conditions were the same as in Fig. 1. ●- - -●, total ATPase activity; ○—○, Mg^{2+}-ATPase.

Fig. 7. Liberation of P_i as a function of K^+ concentration in the presence of 60 mM Na^+. ATP concentration was 3 mM. Other conditions were the same in Fig. 1. ●- - - -●, total ATPase activity; ○—○, Mg^{2+}-ATPase.

The half-maximal activation reported here for Na^+ (4 mM) is less than the value of approx. 8 mM reported by HOKIN[8] for particulate fractions of goose salt gland and the value of 12.5 mM reported by BONTING *et al.*[7] for the salt glands of the herring gull. The half-maximal activation for K^+ (0.8 mM) however, is mid-way between the values of approx. 0.3 and 1.5 mM reported by these authors, respectively.

Development of (Na+-K+)-ATPase and Mg2+-ATPase in the salt gland of ducklings maintained on salt- and fresh-water regimens

The effect of a salt load on the salt gland was readily apparent after 2 days. During this period, the salt glands of salt-loaded ducks (experimental birds) doubled in weight, whereas the weight of glands of birds maintained on fresh water (control birds) doubled within 5 days. Moreover, when gland weight is expressed per 100 g body weight, the experimental birds showed a dramatic increase in this ratio during the first 2 days of salt loading, followed by at least a 13-day period during which the ratio remained constant (Fig. 8). Only after 16 days on the salt diet did the increase in body weight begin to outstrip the increase in gland weight. In control birds, however, this ratio decreased throughout the experimental period (Fig. 8).

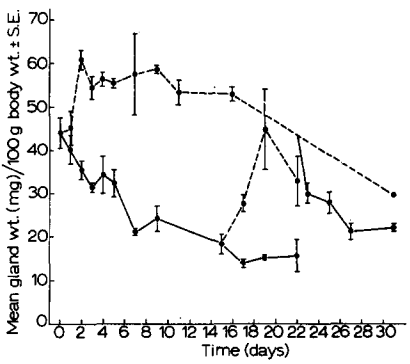


Fig. 8. The effect of NaCl and fresh-water regimens on the mean weight (mg) of the salt glands relative to the mean 100 g body weight on various days. The vertical lines indicate the standard error of the mean. ●—●, birds maintained on a fresh-water regimen; ●- - -●, birds maintained on a 1% aqueous NaCl regimen.

The increase in gland weight of the experimental birds was parallelled by an increase in ATPase activity. The activity of Mg^{2+}-ATPase and (Na^+-K^+)-ATPase reached maximal levels after 9 days on the salt diet, the former increasing by approx. 2.5 times and the latter by more than 5.5 times (Figs. 9 and 10). In contrast, control birds showed little, if any, elevation in ATPase activity above that on the first day of the experiment (Figs. 9 and 10). The results are similar when enzyme activity is expressed per mg of protein. Both enzymes in the experimental birds reached maximal levels of activity by 9 days, the Mg^{2+}-ATPase activity increasing by a factor of 1.8 and the (Na^+-K^+)-ATPase activity increasing by a factor of 4.1 (Figs. 11 and 12). Control birds retained the same basal level of ATPase activity throughout the experimental period.

To determine whether the salt-induced levels of ATPase activity are stable in

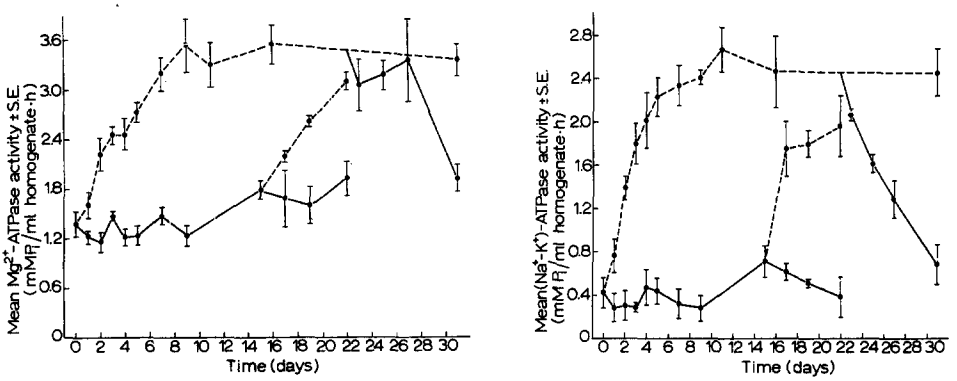


Fig. 9. The effect of NaCl and fresh-water regimens on the mean Mg^{2+}-ATPase activity on various days. The vertical lines indicate the standard error of the mean. The assay conditions were the same as those described in Fig. 1; the concentration of ATP was 3 mM. ●—●, birds maintained on a fresh-water regimen; ●- - - -●, birds maintained on a 1% aqueous NaCl regimen.

Fig. 10. The effect of NaCl and fresh-water regimens on the mean (Na^+-K^+)-ATPase activity on various days. The vertical lines indicate the standard error of the mean. The assay conditions were the same as described in Fig. 1; the concentration of ATP was 3 mM. ●—●, birds maintained on a fresh-water regimen; ●- - - -●, birds maintained on a 1% aqueous NaCl regimen.

the absence of a salt load, a group of salt-water birds was placed on fresh water on the 22nd day of the experiment. Mg^{2+}-ATPase showed only a small decrease in activity during the first 5 days on fresh water. Between the fifth and ninth day, however, the enzyme activity decreased by more than 30% (Figs. 9 and 11). (Na^+-K^+)-ATPase activity, however, decreased gradually during the period of salt deprivation

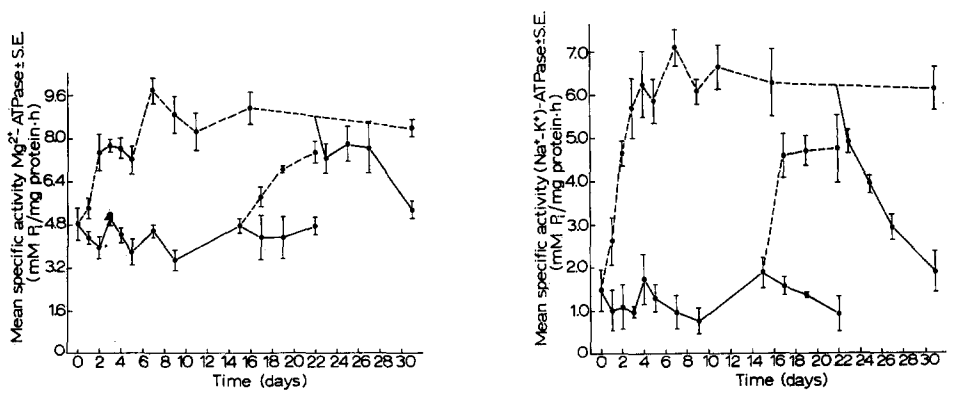


Fig. 11. The effect of NaCl and fresh-water regimens on the mean specific activity of Mg^{2+}-ATPase on various days. The vertical lines indicate the standard error of the mean. The assay conditions were the same as described in Fig. 1; the concentration of ATP was 3 mM. ●—●, birds maintained on a fresh-water regimen; ●- - - -●, birds maintained on a 1% aqueous NaCl regimen.

Fig. 12. The effect of NaCl and fresh-water regimens on the mean specific activity of (Na^+-K^+)-ATPase on various days. The vertical lines indicate the standard error of the mean. The assay conditions were the same as described in Fig. 1; the concentration of ATP was 3 mM. ●—●, birds maintained on a fresh-water regimen; ●- - - -●, birds maintained on a 1% aqueous NaCl regimen.

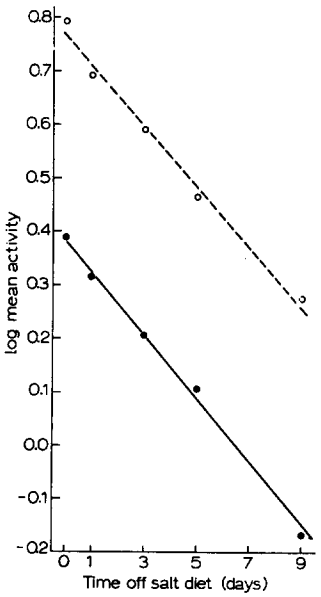


Fig. 13. The effect of salt deprivation, expressed as a logarithmic function, on the mean (Na+-K+)-ATPase activity in homogenates of salt glands from ducks previously maintained on the NaCl regimen. The plot is from the data shown in Figs. 10 and 12. ●—●, log of mean (Na+-K+)-ATPase activity (mM P_i per ml homogenate per h); ○- - -○, log of mean specific activity (mM P_i per mg protein per h) of (Na+-K+)-ATPase.

(Figs. 10 and 12) and by 9 days reached the levels observed in control birds maintained exclusively on fresh water. The decrease in activity of 70% during the 9-day period was logarithmic (Fig. 13) and the half-life of the enzyme was approx. 5 days. During the first day on the fresh-water regimen, the ratio of gland weight to body weight decreased sharply, but thereafter the decline was less rapid (Fig. 8).

When birds, maintained on fresh water for 15 days, were placed on the salt-water regimen, the activity of Mg^{2+}-ATPase rose almost linearly and increased by a factor of 1.6 within 7 days (Figs. 9 and 11). (Na+-K+)-ATPase, on the other hand,

TABLE I

THE EFFECT OF VARIOUS SALT-WATER REGIMENS ON THE MEAN ACTIVITY OF Mg^{2+}-ATPase AND (Na+-K+)-ATPase IN SALT GLAND HOMOGENATES AFTER 9 DAYS OF SALT LOADING

Birds maintained on NaCl, KCl, $MgCl_2$ or fresh-water regimens for 9 days were assayed for ATPase activity in the assay media described in Fig. 1; the concentration of ATP was 3 mM.

Salt in regimen	*Mean Mg^{2+}-ATPase activity at 9 days ± S.E. (mM P_i/ml homogenate · h)*	*Relative per cent of Mg^{2+}-ATPase*	*Mean (Na+-K+)-ATPase activity at 9 days ± S.E. (mM P_i/ml homogenate · h)*	*Relative per cent of (Na+-K+)-ATPase*
NaCl	3.540 ± 0.317	100.0	2.410 ± 0.058	100.0
KCl	2.427 ± 0.245	68.6	1.107 ± 0.173	45.9
$MgCl_2$	2.130 ± 0.041	60.2	1.200 ± 0.140	49.8
None	1.240 ± 0.122	35.0	0.280 ± 0.116	11.6

increased by a factor of 2.4 within 2 days and rose only gradually thereafter (Figs. 10 and 12). This elevation in ATPase activity was reflected by an increase in the ratio of gland weight to body weight (Fig. 8), although this ratio began to decrease between the fourth and seventh day on the salt-water regimen. A concurrent decrease in birds maintained on an exclusively salt-water diet was apparent also at this time.

Since nasal secretion is not a specific response to Na^+ (refs. 11, 21), the increase in ATPase activity in the nasal glands of salt-loaded ducklings may also not be specific to Na^+. Accordingly, two groups of ducklings were raised on either a KCl or on a $MgCl_2$ regimen and their salt glands were assayed for ATPase activity 9 days later. By then, birds raised on a NaCl regimen have reached maximal levels of ATPase activity (Figs. 9–12). These cations increased the levels of ATPase activity to the

TABLE II

THE EFFECT OF VARIOUS SALT-WATER REGIMENS ON THE MEAN SPECIFIC ACTIVITY OF Mg^{2+}-ATPase AND (Na^+-K^+)-ATPase IN SALT GLAND HOMOGENATES AFTER 9 DAYS OF SALT LOADING

The mean activity of Mg^{2+}-ATPase and of (Na^+-K^+)-ATPase reported in Table I is expressed in terms of specific activity.

Salt in regimen	*Mean specific activity of Mg^{2+}-ATPase at 9 days ± S.E. (mM P_i/mg protein · h)*	*Relative per cent of Mg^{2+}-ATPase*	*Mean specific activity of (Na^+-K^+)-ATPase at 9 days ± S.E. (mM P_i/mg protein · h)*	*Relative per cent of (Na^+-K^+)-ATPase*
NaCl	8.86 ± 0.65	100.0	6.08 ± 0.29	100.0
KCl	6.24 ± 0.45	70.4	2.88 ± 0.51	47.4
$MgCl_2$	5.75 ± 0.13	64.9	3.22 ± 0.33	53.0
None	3.46 ± 0.37	39.1	0.75 ± 0.30	12.3

same extent (Tables I and II), although neither was as effective as NaCl. This observation correlates well with the data of GOERTEMILLER AND ELLIS[21], showing that a number of cations elicit some hypertrophy of the salt gland although Na^+ is most effective.

DISCUSSION

Numerous studies demonstrate the association of (Na^+-K^+)-ATPase with active transport[1–9]. The enzyme, however, has not been studied extensively in the context of developing systems, although KLEIN[22] showed that in chick ventricles there is a five-fold increase in the specific activity of the enzyme during the first 12 days of embryonic life. The salt glands of marine birds are unique organs for this kind of study since their development and differentiation is stimulated by ingested salts[18,19,21].

BONTING *et al.*[7] found that salt gland homogenates of herring gulls maintained on fresh water for 7 weeks after capture had 49% less (Na^+-K^+)-ATPase activity and 36% less Mg^{2+}-ATPase activity than those of newly captured birds. The experiments reported here indicate that the salt glands of domestic ducks maintained on fresh water have 88% less (Na^+-K^+)-ATPase and 65% less Mg^{2+}-ATPase than the maximal ATPase activities of birds raised on a salt-water regimen (Tables I and II). The two different species used in these experiments may explain the quantitative

differences that exist between these results and those reported by BONTING *et al.*[7]. In the domestic duck, maintained on salt water, (Na^+-K^+)-ATPase activity declined by 70% from the maximal level to control levels after only a 9-day period of salt deprivation (Figs. 10 and 12), whereas in the gull, this activity fell only 49% after 7 weeks on fresh water.

In contrast, HOKIN[8] was unable to show any difference in the levels of either enzyme in salt gland homogenates of gulls raised either on salt water or on fresh water. HOKIN did not state whether there was a noticeable difference between the gland weights of experimental and control gulls. Since gulls are capable of excreting up to 300 mequiv/l of Na^+ through the kidney[23], a 1.5% salt load (approx. 260 mequiv/l) may be below the threshold for inducing extrarenal secretion and for stimulating a concomitant rise in ATPase levels. In the experiments reported here, birds were maintained on only a 1% solution of NaCl which is presumably within the range of renal competence; however, the ducks were fed a starting mash containing 1% salt by weight. The total salt intake then, was much greater than that derived from the 1% NaCl drinking water and was sufficient to cause both salt gland growth and nasal secretion.

BONTING *et al.*[7] suggested that reduced Na^+ intake by gulls previously maintained on a NaCl regimen led to eventual irreversible degeneration of the salt gland and loss of (Na^+-K^+)-ATPase activity. The loss of this activity is characteristic of the salt gland of the duck as well (Figs. 10 and 12), although it seems unlikely that salt deprivation leads to gland degeneration since birds, maintained on fresh water for 15 days, quickly increased their (Na^+-K^+)-ATPase activity in response to a salt load (Figs. 10 and 12) and were capable of extrarenal secretion after several days on the salt-water regimen.

It is not clear from the literature whether Mg^{2+}-ATPase and (Na^+-K^+)-ATPase are different enzymes. For example, JARNEFELT[24] showed that in the microsomal fraction of rat brain, Mg^{2+}-ATPase could be converted to (Na^+-K^+)-ATPase by treatment with deoxycholate. SKOU AND HILBERG[25] demonstrated that treatment of ox-brain preparations with various sulfhydryl-blocking reagents caused a decrease in Mg^{2+}-ATPase activity and an increase in (Na^+-K^+)-ATPase activity. Such evidence indicates the presence of a single enzyme. On the other hand, HOFFMAN AND RYAN[26], in experiments on erythrocyte ghosts, found that the substrate site was located on the extracellular side of the plasma membrane for Mg^{2+}-ATPase and on the cytoplasmic side of the plasma membrane for (Na^+-K^+)-ATPase. In the present report, the rates of increase of Mg^{2+}-ATPase and (Na^+-K^+)-ATPase in response to a salt load were clearly different (Figs. 9–12). After 9 days on the salt diet, the specific activity of (Na^+-K^+)-ATPase had increased more than twice as fast as Mg^{2+}-ATPase. Moreover, both enzymes showed different rates of degradation following salt deprivation (Figs. 9–12). These results suggest that there are two different ATPases in these salt gland preparations.

The demonstration of a striking increase in Mg^{2+}-ATPase and (Na^+-K^+)-ATPase activity in response to salt loading is consistent with the process of enzyme induction. Although further experiments are necessary to substantiate this conclusion, enzyme activation or removal of an inhibitor seem to be unlikely alternatives in view of the rather long time period necessary to reach maximal levels for both ATPases.

Lastly, the increase in ATPase levels due to Na^+ loading was not cation-specific

(Tables I and II). However, these quantitative differences in the ATPase levels of birds maintained on a NaCl, KCl or $MgCl_2$ regimen may reflect differences in the amount of salt water ingested by the birds since this factor was not controlled. The suggestion by SCHMIDT-NIELSEN, JORGENSEN AND OSAKI[11] that nasal secretion is due to a general osmotic load correlates well with the non-specific cation induction of ATPase activity reported here. Since extrarenal secretion is affected by the adrenal gland[15], it seems likely that ATPase induction is also under hormonal control. It would be valuable, in this respect, to study the induction of (Na^+-K^+)-ATPase activity in adrenalectomized birds.

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